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Genetic transformation of major cereal crops

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ABSTRACT Of the more than 50,000 edible plant species in the world, at least 10,000 species are cereal grains. Three major cereal crops, rice (*Oryza sativa*), maize (*Zea mays*), and wheat (*Triticum* sp.), provide two-thirds of the world's food energy intake. Although crop yields have improved tremendously thanks to technological advances in the past 50 years, population increases and climate changes continue to threaten the sustainability of current crop productions. Whereas conventional and marker-assisted breeding programs continue to play a major role in crop improvement, genetic engineering has drawn an intense worldwide interest from the scientific community. In the past decade, genetic transformation technologies have revolutionized agricultural practices and millions of hectares of biotech crops have been cultured. Because of its unique ability to insert well-characterized gene sequences into the plant genome, genetic engineering can also provide effective tools to address fundamental biological questions. This technology is expected to continue to be an indispensable approach for both basic and applied research. Here, we overview briefly the development of the genetic transformation in the top seven cereals, namely maize, rice, wheat, barley (*Hordeum vulgare*), sorghum (*Sorghum* sp.), oat (*Avena sativa*), and millets. The advantages and disadvantages of the two major transformation methods, *Agrobacterium tumefaciens*-mediated and biolistic methods, are also discussed.

KEY WORDS: *Agrobacterium tumefaciens, biolistic gun, cereal, genetic transformation*

Introduction

Cereals belong to the monocotyledonous family of Poaceae (Fig. 1). The first cereal grains were domesticated back in the Neolithic. The appearance of primitive agriculture led to transition and replacement of a nomadic hunting-gathering lifestyle to a more settled and agrarian-based society (Childe, 1954). Since these historical times, the cereal production has been intensified and, most dramatically, since the mid-20th century thanks to plant breeding technology and ameliorated farming practices. In the last decade, modern science and technology have further improved crop yields. The world's total cereal production has increased from over 1,900 million tons in 2002 to over 2,300 million tons in 2012 according to the Food and Agriculture Organization of the United Nations (FAO, 2013). Yet, to meet the ever augmenting food demand of the growing population, the cereal production still needs to be enhanced. According to the 2012 production data (FAO, 2013), the top seven cereal crops, namely maize (*Zea mays*), rice (*Oryza sativa*), wheat (*Triticum* sp.), barley (*Hordeum vulgare*), sorghum (*Sorghum* sp.), oat (*Avena sativa*), and millets, ranging from 883 million tons for maize to 27.7 million tons for millet, provide approximately two-thirds of the world food supply (Borlaug, 2002). Therefore, it is imperative that the yield of these cereals be increased or at least stabilized for the world's food security purpose.

Currently, the major limitations in cereal production faced by farmers and scientists are abiotic and biotic stresses that, when combined, can lead to 30%-60% yield loss each year all over the world (Dhlamini *et al.,* 2005). Drought, waterlogging, salinity, and temperature represent the major abiotic threats, whereas biotic stresses that include bacterial, viral and fungal pathogens, weeds, and pests (such as cereal aphids, fruit flies, and worms) have caused historically severe yield reductions. Thanks to conventional breeding, the resistance/tolerance to both abiotic and biotic stresses has advanced greatly with a spectacular yield increase during the last century. For example, double-cross hybrids (hybrids

Abbreviations used in this paper: BAP, 2-benzylaminopurine; *bar*, phosphinothricin acetyltransferase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; *gfp*, green fluorescence protein gene; *gus*, β-glucuronidase-encoding gene; Ha, hectare; Hg, hectogram; *hpt*, hygromycin phosphotransferase-encoding gene; IE, immature embryo; MDE, microspore-derived embryo; *nptII*, neomycin phosphotransferase II-encoding gene; PEG, polyethylene glycol; *pmi*, phosphomannose isomerase-encoding gene; PPT, phosphinothricin; SMC, shoot meristematic culture; TF, transformation frequency.

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generated by crossing two single-cross hybrids) in maize has enhanced the yield from ~1.5 ton/ha to ~3.5 ton/ha between 1930 and 1960, whereafter single crosses were introduced to produce corn hybrids that increased the corn yield to over 8 ton/ha by the end of last century (Lamkey and Edwards, 1999). Another example is the green revolution of the 1960s that saved a billion people from starvation owing to the development of semi-dwarf and disease-resistant wheat varieties with a high-yielding potential (Borlaug, 2002). As a population of eight billion people is predicted by 2025, i.e. one billion more than the current one (http://www.worldometers.info/wordl-population/), a 60% improvement in cereal production is estimated necessary to meet such a food demand, which puts a huge pressure on yield, given that the arable surface remains the same. Therefore, in addition to conventional breeding, biotechnology is a complementary tool to overcome hunger, because it can discover agronomically important genes for stress resistance and yield improvement and, consequently create new genetic resources and germplasms through mutations and genetic transformation.

Genetic transformation is a technology that introduces genetic elements, such as DNA, directly into a cell's genome, thus generating products that cannot be achieved through conventional breeding that does not allow insertion of exogenous genes from unrelated species. A well-known example is the widely grown biotech *Bt* corn, which is a transgenic line expressing a bacterial gene from *Bacillus thuringiensis* that provides resistance against the European corn borer (*Ostrinia nubilalis*) (Ostlie *et al.,* 1997). Numerous methods exist for introducing DNAs into the plant genome. Most dicotyledonous plants, such as tobacco (*Nicotiana tabacum*) can be transformed readily by the soil bacterium and plant pathogen, *Agrobacterium tumefaciens* (Cheng *et al.,* 2003). However, because cereals are not natural hosts of *Agrobacterium* and are recalcitrant to this method, no genetic transformation had been reported before 1993 (Chan *et al.,* 1993). In the meantime, a number of direct DNA transfer methods, such as particle bombardment (biolistic or gene gun), electroporation, and microinjection, had been developed to overcome the limitation of the *Agrobacterium*-mediated method. Thanks to worldwide research efforts from both academic and industrial laboratories in the past two decades, most cereal crops, especially the top seven, can be transformed genetically. This technological advancement provides tremendous opportunities for basic and applied research in these cereal crops. Here, we review the history as well as the current status of the top seven cereal crops, focusing on biolistic and *Agrobacterium*-mediated delivery systems. While writing this review, we noticed that the definition of "transformation frequency" (TF) was not consistent in the literature, especially in the early publications on genetic transformation: some papers defined TF as the number of herbicide-resistant or antibiotic-resistant tissue per 100 infected or bombarded targeting materials, whereas others calculated TF as the number of fertile transgenic plants per 100 starting materials. Therefore, even though we cite the TFs reported in those publications, we do not compare them directly.

Maize (*Zea mays*)

Domesticated from teosinte more than 5,000 years ago, maize is the most important food/feed crop and most produced grain among the world top seven cereals (Gewin, 2003; Wang *et al.,* 2009). The world average yield of maize was increased from 19423 hectogram (Hg)/hectare (Ha) in 1961 to 51847 Hg/Ha in 2011 (FAO, 2013), nearly tripled in the past 50 years. The world maize harvest area also expanded from 106 million Ha to 170 million Ha between 1961 and 2011 (FAO, 2013). The extraordinary yield increase can be attributed to the improved germplasms, especially hybrids, through breeding programs, as well as intensified farming practice (Scheffler *et al.,* 2008).

The first maize transformation attempts can be traced back as early as 1966, when genomic DNA had been injected directly into apical meristems of developing maize seedlings (Coe and Sarkar, 1966). However, the experiment was unsuccessful because no expected phenotypical changes were observed. Twenty years later, exogenous DNA could be introduced into maize by means of *Agrobacterium tumefaciens* (Grimsley *et al.*, 1986). Since the first maize transformation by the biolistic gun method (Gordon-Kamm *et al.*, 1990) and the *Agrobacterium*-mediated method (Ishida *et al.*, 1996), maize is now routinely transformed for a few genotypes in many laboratories around the world. For a successful maize transformation system, a number of factors, including tissue cultures, delivery systems, and selectable markers/agents need to be optimized.

Tissue culture

Maize transformation depends both on genotype and tissue. The ideal target tissues or cells should be both transformable

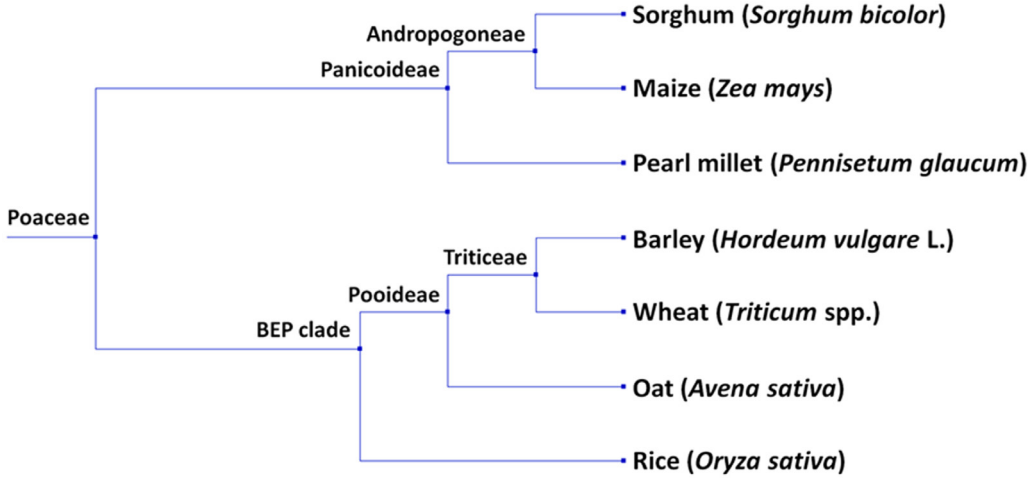

Fig. 1. Phylogenetic relationships among seven crop plants. *The phylogenetic tree is built based on the Taxonomy database of the National Center for Biological Information (http://www.ncbi.nlm.nih.gov/taxonomy), which contains all sequences represented in the International Nucleotide Sequence Database Collaboration (INSDC), including the GenBank, ENA (EMBL) and DDBJ databases. The graph was generated by software Notung 2.6 (http://www.cs.cmu.edu/~durand/Notung/).*

and regenerable, but not all maize cells can be regenerated. In fact, many abundant and readily available maize tissues, such as leaves, roots, and mature seeds are not amenable for *in vitro* culture and regeneration.

In the early days, protoplast cultures were considered for transformation because DNA could be introduced directly into protoplasts via polyethylene glycol (PEG), electroporation, or *Agrobacterium*-mediated methods. The first successful transgenic maize was generated from protoplasts of embryogenic suspension cultures of the inbred maize line A188 (Rhodes *et al.,* 1988), but none of the 38 plants from 10 transformed lines could set seed. In 1993, the first fertile transgenic maize was reported from protoplasts that had been isolated from embryogenic suspension cultures of genotype HE/89 (Golovkin *et al.,* 1993; Omirulleh *et al.,* 1993). More than 60% of the transgenic plants could set seed. Because of the strong genotypic dependency and elaborated tissue preparation, maize protoplasts from embryogenic callus cultures are currently not the preferred system for stable transformation, but maize protoplasts from leaf material can be a useful system for many transient studies (Sheen, 1996).

Immature embryos (IEs) and IE-derived embryogenic cultures are alternative target tissues to protoplasts for maize transformation. In 1990, the first fertile transgenic maize was obtained from embryogenic suspension cultures derived from IEs of the maize hybrids A188XB73 or B73XA188 (Fromm *et al.,* 1990; Gordon-Kamm *et al.,* 1990). Currently, most maize transformation laboratories do not use IE-derived suspension cultures or callus cultures, but, instead, directly IEs as target tissues. IEs harvested 10-12 days after pollination are dissected and transformed either by particle bombardment or *Agrobacteruim* infection. These embryos are cultured in media containing selection agents and transformed cells that survived selection are bulked up, regenerated, and grown to maturity for seed setting. The TFs for tissue culture-amenable maize genotypes, such as inbred A188 or Hi-II (a hybrid with A188 and B73 background created specifically for *in vitro* culture purpose) range from 5% to 50% (Ishida *et al.,* 1996; Zhao *et al.,* 2001; Frame *et al.,* 2002).

The major limitation of maize transformation based on IEs and IE-derived callus cultures is its genotype dependence. Two types of embryogenic callus cultures have been successfully used: Type II callus that is more friable than Type I callus in the culture morphology and is more suitable for selection, regeneration, and culture suspension (Songstad, 2010) and the Hi-II genotype that forms Type II callus is one of the most successful genotypes for maize transformation (Zhao *et al.,* 2001; Frame *et al.,* 2002). Some maize inbred lines form Type I callus (Frame *et al.,* 2006; Songstad, 2010).

To overcome the genotype dependence in callus culture production, other tissue types have been investigated. One explant type is shoot meristem cells, because they can generate shoots without a callus stage, providing the opportunity to transform elite genotypes that are often recalcitrant to callus induction. Although these attempts were promising, the meristem tissue-based transgenic plants were chimeric, the TFs were low, and, most importantly, the ability of induce shoot multiplication at high frequencies from excised meristems remained genotype dependent. Developing shoot meristem of IEs were isolated from a number of inbred maize lines and bombarded with DNA carrying the visual β-glucuronidase (*gus*) and the selectable neomycin phosphotransferase II (*nptII*) marker genes. The bombarded tissues were cultured for shoot proliferation on media containing cytokinin (1 mg/L 2-benzylaminopurine [BAP]) and 50-100 mg/L kanamycin sulfate. A good level of GUS activity could be observed in the tissues 4 weeks after bombardment, but the TFs in the three experiments varied between 0.4% to 2.5% (Lowe *et al*., 1995).

Another explant type is the maize male gametophyte, such as pollen. There are two advantages in using maize pollen: no tissue culture procedures are needed and maize pollen is abundant, promising large numbers of independent events. The first maize pollen transformation experiments were conducted by mixing maize pollens with genome DNA isolated from a phenotypically different donor plant to generate seeds (De Wet *et al.,* 1985; Ohta, 1986). The bombardment method was also used in maize pollen transformation (Horikawa *et al.,* 1997). Unfortunately, no concrete molecular confirmation and progeny data analysis were reported and, in general, the low frequencies or lack of reproducibility have prevented these tissues from becoming routine targeting tissues.

Although IEs are the most successful target material for maize transformation, for many public institutions, it can be a challenge to obtain year-round large supplies. Therefore, mature seeds or seedling-derived cultures are desired alternatives. In a shoot multiplication protocol for over 20 maize inbreds (Zhong *et al*., 1996), shoot-tip clumps from 7-day-old seedling segments of mature seeds were bombarded with DNA carrying the gene coding for bialaphos (phosphinothricin [PPT] with two alanine residues isolated from the bacterium *Streptomyces hygroscopicus*) (*bar*) and were cultured on media without any selection for one month. Subsequently, selection and regeneration were carried out on media containing 5-10 mg/L of glufosinate. The recovery of the glufosinate-resistant shoot-tip clumps was between 1% and 6.7%.

Alternatively, leaf explants can be readily prepared from germinating maize seedlings, which is less laborious than isolating the IEs. Callus induction and plant regeneration from leaf explants have been demonstrated (Conger *et al.,* 1987, Ray and Ghosh, 1990). A successful leaf-based maize transformation was reported in 2007 via the biolistic method (Ahmadabadi *et al.,* 2007). Sidorov *et al.* (2006) reported the first successful transformation by an *Agrobacterium*-mediated method using Type I callus culture induced from nodal sections of seedlings. They used a C58-derived *Agrobacterium* strain ABI that harbored binary vectors containing the visual marker green fluorescence protein (GFP) gene and either the *nptII* or the *bar* gene as selectable marker. The transformation was confirmed by Southern blot and the TF ranges from 2 to 11%, depending on cultivars (Sidorov *et al.,* 2006).

Delivery systems

For maize transformation, the main DNA deliver systems can be classified into direct DNA transfer and *Agrobacterium*-mediated methods. The major methods of direct DNA transfer include electroporation (Rhodes *et al.,* 1988), PEG incubation (Golovkin *et al.,* 1993), silicon carbide whiskers (Frame *et al.,* 1994), and particle bombardment (Gordon-Kamm *et al.,* 1990).

The most effective direct DNA delivery system is the particle bombardment (or gene gun). In fact, the first transgenic maize plants were generated by means of a gene gun PDS1000/He (the biolistic particle delivery system of BioRad [Hercules, CA, USA]) (Gordon-Kamm *et al.,* 1990). This method remains the method of choice in most laboratories (Sanford *et al*., 1993). In the bombard-

ment system, the DNA fragments carrying the gene of interest are coated on micro size particles, such as gold or tungsten. These particles are propelled by helium gas that provides the kinetic energy to penetrate into the cells after crossing the cell walls. Recently, the gene gun was utilized also to deliver chemicals and proteins loaded with nanoparticles to plant cells (Torney *et al.,* 2007; Martin-Ortigosa *et al.,* 2012).

The *Agrobacterium*-mediated gene delivery system was unsuccessful until 1996, when a superbinary vector system was designed, in which, extra copies of the *Agrobacterium* virulence (*vir*) genes (*virB*, *virC,* and *virG*) (Hiei *et al.,* 1994) were included (Ishida *et al.,* 1996). Since then, standard binary vectors (non-superbinary) have been used to transform both the Hi-II genotype (Frame *et al.,* 2002; Vega *et al.,* 2008) and the inbred B104 (Frame *et al.,* 2006). The major advantage of the *Agrobacterium*-mediated gene delivery system is that it typically inserts fewer transgene copy numbers than the biolistic gun method, thus reducing the probability of gene silencing caused by multiple transgene copies (Tang *et al.,* 2007). Today, the biolistic gun and *Agrobacterium* are the two most efficient and popular methods for genetic transformation of maize.

Selectable markers

A selectable marker gene is introduced to differentiate transformed cells from a population of untransformed cells and is typically cotransformed with the gene of interest. Most selectable markers are antibiotics, such as hygromycin and kanamycin, or herbicides, such as glyphosate and PPT. The first transgenic maize was obtained with PPT (Gordon-Kamm *et al.,* 1990) that is considered as efficient and is a widely used selection system (Fromm *et al.,* 1990, Gordon-Kamm *et al.,* 1990; Ishida *et al.,* 1996; Zhao *et al.,* 2001; Frame *et al.,* 2002, 2006). The PPT selection system contains genes coding either for bialaphos *(bar*), PPT acetyltransferase (*pat)* isolated from the bacterium *Streptomyces viridochromogenes,* or glufosinate (an ammonium salt of PPT). Among the three selective agents, bialaphos is the most efficient to transform the hybrid Hi-II and some other inbred lines (Dennehey *et al.,* 1994).

Another herbicide selection system is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)/glyphosate. EPSPS is a key enzyme that synthesizes chorismate-derived aromatic amino acids in plants and can be inhibited by glyphosate, the active ingredient in the herbicide Roundup® (Monsanto, St. Louis, MO, USA). A mutated EPSPS-encoding gene of maize producing a modified enzyme resistant to glyphosate inhibition had been reported to be an efficient selectable marker for the production of fertile transgenic maize plants (Howe *et al.,* 2002) that regenerated from the glyphosate selection medium and were tolerant to Roundup®.

The kanamycin selection system consists of the *nptII* gene and the antibiotic kanamycin (or its analogs geneticin G418 or paromomycin) and has been used in early maize transformation assays (Rhodes *et al.,* 1988; Gould *et al.,* 1991; D'Halluin *et al.,* 1992; Lowe *et al.,* 1995). Although growth of single maize cells can be inhibited by kanamycin, large-sized chunks of maize cells are less sensitive to this antibiotic (Wang *et al.,* 2009). The typically used concentration of kanamycin (including G418 and paromomycin) is 50 to 200 mg/L (Rhodes *et al.,* 1988; Gould *et al.,* 1991; D'Halluin *et al.,* 1992; Lowe *et al.,* 1995). One of the factors that contributed to the ineffectiveness of this system might be the high endogenous resistance to kanamycin and G418 of maize tissues (Gordon-Kamm *et al*., 1990).

Compared to kanamycin, maize is more sensitive to hygromycin. The hygromycin selection system (with the hygromycin phosphotransferase *hpt* gene from *Escherichia coli* and hygromycin as selection agent) has been applied for both particle bombardment and *Agrobacterium*-mediated maize transformation (Walters *et al.,* 1992; Ishida *et al.,* 1996). Despite the effective inhibition of the cellular growth at 30 mg/L hygromycin when maize cells are not transformed, the TF was 50% or 30% lower than that of the PPT system (Ishida *et al.,* 2007).

Alternative selectable marker genes have been tested for positive selection in maize. Protoporphyrinogen oxidase is an important enzyme in the chlorophyll/heme biosynthetic pathway and oxidizes protoporphyrinogen IX to protoporphyrin IX, which is essential for photosynthetic organisms (Smith *et al.,* 1993). When protoporphyrinogen is repressed by protoporphyrinogen inhibitors, such as butafenacil, protoporphyrinogen IX will accumulate and cause membrane damage and cell death (Smith *et al.,* 1993). Thus, butafenacil can be considered as an herbicide and butafenacil-resistant plants can be generated by selecting resistant mutants or by overexpressing native plant protoporphyrinogen genes. A mutated *Arabidopsis thaliana* protoporphyrinogen gene that was highly tolerant to butafenacil has been identified and successfully used as a selectable marker for *Agrobacterium*-mediated maize transformation (Li *et al.,* 2003). Another well-tested selection system utilizes the phosphomannose isomerase (*pmi*) gene as selectable marker and mannose as selection agent (Negrotto *et al.,* 2000). The *pmi* gene converts mannose-6-phosphate to fructose-6-phosphate (Reed *et al.,* 2001) and, hence, only the transformed cells can exploit mannose as sugar source in the medium.

Rice (*Oryza sativa*)

Rice, an important cereal grain, is one of the most consumed staple foods around the world, especially in Asia. The production of rice grain is the second-highest in the world, next to wheat (FAO, 2013). The worldwide rice yield has increased dramatically from 18693 Hg/Ha to 44037 Hg/Ha from 1961 to 2011 (FAO, 2013). Rice is one of the cereals with the most elaborated *in vitro* tissue culture systems. Like maize, the early transformation work focused on the use of rice protoplasts and the physical delivery methods, such as PEG or electroporation (Toriyama *et al.,* 1988; Zhang and Wu, 1988; Zhang *et al.,* 1988). The first successful production of transgenic rice plants with the biolistic method (Christou *et al.,* 1991) was published one year after the biolistic-mediated transgenic maize (Gordon-Kamm *et al.,* 1990). By using an electric discharge particle apparatus, Christou *et al.,* (1991) introduced DNA-coated gold particles into IEs of several *japonica* and *indica* rice varieties. Bombarded tissues were plated on media containing 50 mg/L hygromycin or 10 mg/L bialaphos for callus induction, selection, and regeneration. Southern hybridization and progeny analysis confirmed that the biolistic method could be an efficient system for rice transformation. The system was improved by continuous selection through regeneration and growth stages and by excising resistant cell clusters at an early stage (2 weeks after selection) (Li *et al*., 1993). Hundreds of transgenic plants for both *japonica* and *indica* varieties were obtained from IEs or IE-derived calli.

Although biolistic-mediated rice transformation protocols were reported before the *Agrobacterium*-mediated protocols, nowadays more than 80% of the transgenic rice is transformed

by the *Agrobacterium* method (Hiei and Komari, 2008). The first *Agrobacterium*-mediated transgenic rice was obtained with IEs as explants and G418 as selection agent (Chan *et al.,* 1993). In 1994, in a milestone work for *Agrobacterium*-mediated transformation of a number of *japonica* rice cultivars (Hiei *et al.,* 1994), various rice tissues, including shoot apices, root segments, scutella, IEs, root callus, scutellar callus, and suspension cell cultures induced from mature seed scutella, were infected with *Agrobacterium* strains EHA101 or LBA4404 carrying either plasmid pIG121Hm (standard binary vector) or pTOK233 (superbinary vector). In both plasmids, the *hpt* gene was the selection marker and the *gus* gene the reporter marker. By comparing the percentage of explants displaying proliferated cells to the total inoculated explants, 3 weeks after cultivation on the selection medium, scutella-derived callus cultures gave the best resulsts (23% explants), followed by suspension cells from scutella (9%) and IEs (6%). The strain/vector combination test indicated that the frequency of positive callus pieces was the highest with the superbinary LBA4404/pTOK233 system. Hundreds of transgenic rice plants were obtained within 4 months, with a TF ranging from 12.8% to 28.6%. Critical factors, such as addition of acetosyringone to the cocultivation media and incubation at 22-28°C were also key to the success of the method (Hiei *et al.,* 1994).

To shorten the transformation time to avoid somaclonal variation caused by the *in vitro* culture, mature seed-derived calli from *japonica* cv. Nipponbare and Kitaake were used as explants and hygromycin as selection agent (Toki, 1997). The tissue culture duration was reduced further (Toki *et al.,* 2006) by preculturing the scutellar tissues from mature seeds of *japonica* cv. Nipponbare for 1 to 5 days before the *Agrobacterium* infection. As a result, the transgenic plants were ready for transfer to soil within 45 days of culture.

The *Agrobacterium*-mediated method was also tested for *indica* and *javanica* (now known as tropical *japonica*) rice. Using 3-week-old callus derived from mature embryos, Rashid *et al.,* (1996) transformed three *indica* rice cultivars with the *Agrobacterium* strain EHA101 harboring the standard binary vector pIG121Hm with the *hpt* gene as a selective marker. Consistently with the findings of Hiei *et al.,* (1994), the calli derived from scutella were excellent explants and the 50 μM acetosyringone in the cocultivation media proved to be indispensable. By modifying the subculture media for callus induction and the differentiation media, the transformation system for *indica* rice was improved (Lin and Zhang, 2005). The mature seed-derived calli of four *indica* cultivars were infected with EHA105/pCAMBIA1301 (containing the *hpt* gene as selective marker). The TF ranged from 8.5% to 23.4%, which is higher than most of the previous reports (Lin and Zhang, 2005).

For the transformation of *javanica* rice by means of the *Agrobacterium*-mediated method, IE-derived, instead of mature seed-derived, callus cultures were infected with the *Agrobacterium* strain and 50 mg/L hygromycin for selection (Dong *et al*., 1996). Interestingly, whereas transgenic *japonica* rice (Taipei 309) could be obtained either with the superbinary or standard binary vectors, only the superbinary vector system could generate transgenic *javanica* rice.

In a rice transformation protocol, in which both IEs and mature seed-derived calli were used to target a wide range of rice varieties (Hiei and Komari, 2008), various *japonica* and *indica* varieties were classified in six genotypic groups based on the recalcitrance levels of tissue culture and transformation. Almost all rice varieties could be transformed with different degrees of success when IEs were used as starting material and infected with the *Agrobacterium* strains LBA4404 or EHA105 carrying the *hpt* gene as selection marker. The number of generated independent transgenic rice plants per IE was 10-18 for *japonica* varieties and *indica* Kasalath and 5-13 for non-Kasalath *indica*. For mature seed-derived callus transformation, 50% to 90% of the calli could generate transformants for the *japonica* varieties and *indica* Kasalath. The time from explant inoculation to transfer of transgenic plants to soil was less than 2 months for the *japonica* varieties and Kasalath and 2.5 months for the non-Kasalath *indica* (Hiei and Komari, 2008). A TF as high as 90% could be achieved in four tested rice varieties (Ozawa, 2009). This significant improvement in TF was largely attributed to the switch from solid cocultivation medium to three layers of filter paper saturated with enriched cocultivation medium (Ozawa, 2009).

Besides the commonly used explants such as the IEs and mature seed-derived calli, other explants, including shoot apices (Park *et al.,* 1996), inflorescences (Dong *et al.,* 2001), and mature embryo-derived green tissues (Cho *et al.,* 2004) were also investigated, but mostly with limited success. For example, the TF was only 0.7% by infecting shoot apices of the tropical *japonica* rice cv. Maybelle with the EHA101 strain carrying the *bar* gene as selection marker (Park *et al*., 1996), whereas a TF of 6.5% was obtained by bombarding 5-month-old to 12-month-old green tissues derived from the mature seed scutellum of *japonica* rice cv. Taipei 309, followed by a hygromycin selection (Cho *et al*., 2004). However, an average of 30% TF was reported by infecting 1- to 3-mm rice inflorescences of the *japonica* rice cv. Taipei 309 with the strain EHA101 carrying both *gus* and *hpt* genes (Dong *et al.,* 2001). Recently, 28-day-old rice calli induced from the leaf bases of 4-day-old rice seedlings from the *indica* rice cv. ADT 43 were infected with the EHA105/pCAMBIA1301 system with a TF of up to 9.33% (Karthikeyan *et al.,* 2011).

Wheat (*Triticum* sp.)

Wheat is grown on more land than any other cereal in the world, with over 217 million Ha of arable land in 2012 (FAO, 2013). It is a gluten-producing cereal that allows the preparation of leavened bread. Wheat provides staple food for over one-third of the human beings and supplies approximately 20% of the calorie intake (Vasil, 2007). Wheat yield has spectacularly improved from 10889 Hg/Ha in 1961 to 31984 Hg/Ha in 2011 (FAO, 2013), since the green revolution, which has saved billions of people from starvation.

Despite its importance, the progress in wheat transformation lagged behind other major cereal crops, due partially to its recalcitrance to *in vitro* regeneration and transformation (Gao *et al.,* 2011). Wheat was first transformed by particle bombardment (Vasil *et al.,* 1992) and the first transgenic wheat resistant to the herbicide Basta® (with PPT as active component) (Bayer Crop Science, Leverkusen, Germany) was obtained by bombarding embryogenic IE-derived callus cultures. The entire process took over one year because of the very time-consuming step of establishing a type C regenerable callus. The callus response rate was low and the regenerated plants showed many somatic variations due to the prolonged culture time (Vasil *et al.,* 1992). In addition, the TF was only 0.2% and the transgenic lines were not fertile (Vasil *et al.,* 1992). The protocol was improved by bombarding the scutellar tissue of IEs or less than 2-month-old embryogenic calli. By using

these explants, the entire transformation process (from IE harvest IEs to the transgenic wheat plants) was reduced to 7-9 months with an average TF of 1-2% (Vasil *et al.,* 1993).

Further efforts shortened the *in vitro* culture time and enhanced the TFs. For example, the time required to produce transgenic plants could be shortened to 8-9 weeks by bombarding osmotically treated IEs that has been cultured for 5-7 days after dissection and, with 3 mg/L bialaphos for selection, a TF as high as 2% in some experiments could be achieved (Altpeter *et al.*, 1996). The age of the donor plant was also found to be critical for biolistic wheat transformation: by using IEs from plants grown for 70, 76, and 85 days, the average TF increased from 0.7% (76 or 85 days) to 5% (70 days, corresponding to 12-14 days post anthesis) (Pastori *et al.,* 2001). This difference was attributed to the likely fluctuation of the phytohormones, such as cytokinins, auxins, and gibberellins, which play an important role in the tissue cultures at that time point (Pastori *et al.,* 2001).

The first success of *Agrobacterium*-mediated transformation was reported for spring wheat Bobwhite (Cheng *et al.*, 1997). Freshly dissected IEs (cultured for 3-4 hours), precultured IEs (cultured for 1-6 days), and embryogenic calli (cultured for 10-35 days) as explants were infected with *Agrobacterium* strain C58 harboring the binary vector pMON18365 that carried the *nptII* gene as selection marker (Cheng *et al.*, 1997). Stable transgenic plants were obtained in 3 months with an average TF of 1.1-1.6%. Following the similar protocol, but with the *bar* gene for selection, Weir *et al.,* (2001) obtained four transgenic events from 218 embryo scutella, with a 1.8% TF. Addition of spermidine to the regeneration medium enhanced the TF (Khanna and Daggard, 2003). Introduction of the superbinary vector pHK21 (in *Agrobacterium* strain LBA4404) carrying an additional set of *vir* genes and the *bar* gene as selection marker into IE-derived calli from spring wheat cv. Veery5, increased the recovery rate of the regenerants to 24.2% compared with 7% from regeneration media without spermidine and, accordingly, the final TF from 1.2% (without spermidine) to 3.9% (with spermidine). However, no transgenic plants were obtained with the standard binary vector pHK22 (without extra set of *vir* genes), suggesting that the superbinary vector was important in the successful transformation (Khanna and Daggard, 2003). The transformation system was optimized by using IEs from four wheat varieties and the AGL1 strain carrying the pGreen-based plasmid with the *bar* gene as selection marker (Wu *et al.*, 2003). The results implied that many factors, such as embryo size, preculture, duration of infection and cocultivation, and acetosyringone and surfactant concentrations, all played a role in a successful transformation. Another system suitable for large-scale wheat transformation was developed by means of the *aroA:CP4* as the selective gene that confers resistance to glyphosate (a major component of the herbicide Roundup®). Glyphosate kills all the nontransgenic plants by inhibiting the biosynthesis of aromatic amino acids. In this system, 4-day-old precultured IEs of spring wheat cv. Bobwhite were used as target tissue for *Agrobacterium* strain C58 infection, with an average 4.4% TF (Hu *et al.*, 2003). Importantly, it took only 50-80 days from isolation of the embryos to transgenic plantlets. An increase from 0.7% to 9.4% TF was obtained by simply desiccating plant tissues until no obvious liquid was observed between the explants after *Agrobacterium* infection, which might suppress the overgrowth of *Agrobacterium* and provide favorable condition for plant cell recovery (Cheng *et al.,* 2003). Additionally, a resting period of 48-72 hours after infection is important for regeneration of transgenic plants from IEs (Cheng *et al.,* 2003; Hensel *et al.,* 2009). Durum wheat varieties have also been successfully transformed by *Agrobacterium*. An average of 3.1% TF in durum wheat cv. Ofanto was reported with a superbinary pGreen/pSoup system (Wu *et al.*, 2008) and was even improved to 6.3% in durum wheat cv. Stewart by increasing the piclorm and acetosyringone concentrations in the infection and/or cocultivation media (He *et al.*, 2010).

Recently, a highly efficient semi-*in planta* method has been described (Risacher *et al.,* 2009). In this system, 16-18 days post anthesis wheat tillers were harvested from growth chambers and the immature seeds were inoculated by syringe injection of the *Agrobacterium* strain EHA105 carrying a binary vector with the *gus* and *nptII* genes. Two to 3 days after the inoculation, the seeds were isolated from ear and surface-sterilized. IEs were dissected from the infected seeds, and placed on medium containing 25 mg/L G418 for induction and selection. Resistant calli could be regenerated 2-3 months after infection. An average of 5% TF (ranging from 1 to 30%) could be achieved with this method for spring wheat NB1 (Risacher *et al.,* 2009).

In addition to commonly used antibiotics and herbicides as selection system, other marker genes, specifically positive selection markers, have been tested in wheat. Cyanamide hydratase is an enzyme which converts cyanamide into urea, hence promotes plant growth (Maier-Greiner *et al.,* 1991). By using a cyanamide hydratase-coding gene isolated from the soil fungus *Myrothecium verrucaria* as selective marker, Weeks *et al.,* (2000) successfully obtained two stable transgenic events, but the TF was only 0.2%. The *pmi* gene was also investigated as selectable marker gene and the TF could reach 20% in spring wheat UC703 by bombardment (Wright *et al.,* 2001).

Barley (*Hordeum vulgare* L.)

Barley is the fourth most important cereals in the world in terms of production. It is mainly used for animal feeding, food, and malting. Barley can adapt readily to different climates and is able to tolerate abiotic stresses, such as cold, drought, salinity, and alkalinity. After many years of domestication, it can now be found in northern Scandinavia, the Himalayan Mountains, monsoon areas, such as India, the Southwest of the United States, Australia, and Niger (Hayes *et al.,* 2003). Breeding efforts in the past 50 years has doubled the barley yield (from 13282 Hg/Ha in 1961 to 27627 Hg/Ha in 2011) (FAO, 2013). Therefore, even with the shrinking planting acreage from 54.4 million Ha in 1961 to 48.6 million Ha in 2011, the world-wide barley production did not change too much (from 7.8 million tons in 1961 and 8.2 million tons in 2011) (FAO, 2013). For researchers, barley is also a good model species for wheat research, due to its smaller and less complicated diploid genome.

As for many other cereal crops, transgenic barley was first achieved through the biolistic delivery method. In the first report on barley transformation, a number of targeting explants, including IEs, IE-derived callus, and microspore-derived embryos (MDEs) of spring barley cv. Golden Promise, were bombarded with the BioRad PDS1000/He delivery system (Wan and Lemaux, 1994). A large number of self-fertile transgenic barley lines were produced by means of the *bar*/bialaphos selection system, with an average TF of 7.9% for IEs and 0.3% for MDEs (Wan and Lemaux, 1994).

One challenge in the barley *in vitro* culture process is the loss

of regenerability and albinism (Kott and Kasha, 1984; Jähne *et al.,* 1991). Indeed, approximately 50% of the transgenic lines from IEs and MDEs and 100% of the lines from young embryogenic callus were albino. As a solution to this problem, addition of BAP (0.01-0.1 mg/L) and copper (5 μM) to the media was found to lead to more regenerable callus and green shoots for the barley variety Golden promise (Cho *et al*., 1998). Furthermore, this improvement could be extended to other recalcitrant barley cultivars, such as Galena and Harrington (Cho *et al.,* 1998).

Three years after the generation of the first biolistic-derived transgenic barley, the *Agrobacterium*-mediated transformation method was reported by Tingay *et al.,* (1997). IEs were used as target tissues and the *Agrobacterium* standard binary vector system carrying the *bar* gene; from over 1,200 infected embryos (subsequently selected on 3 mg/L bialaphos), 54 transgenic lines were obtained, giving a 4.2% TF. Although acetosyringone was not added at the infection stage, embryo axes were removed and the embryos were bombarded with gold particles prior to *Agrobacterium* infection. This practice proved helpful in recovering transformed tissues in the preliminary tests, in which fewer bialaphos-resistant callus were obtained from embryos without any treatment (Tingay *et al.,* 1997). Multiple independent transformants were generated from single embryo explants and used to calculate the TF.

In the first successful *Agrobacterium*-mediated transformation with Australian elite cultivars instead of Golden Promise, IEs from growth chamber-cultivated plants were harvested, isolated, and infected with an *Agrobacterium* strain carrying the standard binary vector system and the *hpt*/hygromycin selection system (Murray *et al*., 2004). This accomplishment was attributed to both the optimal growth conditions of the donor plants (controlled environment) and the use of hygromycin as selective agent. Because the Australian elite cultivars grew more slowly than the Golden Promise cultures, they were more prone to *Agrobacterium* overgrowth, which is detrimental to *in vitro* culture health and growth. The presence of hygromycin in the media seemed to be able to suppress the *Agrobacterium* overgrowth in addition to its function as selective agent, thus fostering the vigorous callus growth in barley transformation. However, the reported TF for the elite cultivars was much lower than that for Golden promise (0.6% versus 4.4-9.2%), probably due to a lower regenerability of the elite cultivars used (Murray *et al.,* 2004).

A breakthrough in the barley Golden Promise transformation was the introduction into IEs via the *Agrobacterium* strain of the pBract serial vectors that contain the *hpt* gene as selection marker and the luciferase (*luc*) gene as reporter gene (Bartlett *et al*., 2008). Addition of copper in the callus induction medium and inclusion of an intermediate culture step (transition medium) before regeneration increased effectively the average TF to as high as 25%. Approximately half of the transgenic lines contained single-copy insertions.

Transformation with alternative target tissues, such as shoot meristematic cultures (Zhang *et al.,* 1999), androgenetic pollen cultures (Jähne *et al.,* 1994; Yao *et al.,* 1997; Kumlehn *et al.,* 2006), *in vitro* cultured ovules (Holme *et al.,* 2008), and mature embryos (Sharma *et al.,* 2005), have also been attempted. Shoot meristematic cultures from germinating seedlings of barley cv. Harrington have been used for the biolistic delivery of the *bar/nptII* and *gus* genes (Zhang *et al.,* 1999). However, the TF was very low because only one fertile transgenic barley plant with stable expression was obtained from all six bombardments from a total of 240 explants (Zhang *et al.,* 1999). Mature embryos also proved to be good targets in both callus induction and regeneration among seven barley cultivars tested (Sharma *et al.,* 2005), hinting at the potential of mature embryos for transformation, but this explant was almost never mentioned in the literature.

Microspores have been considered as one of the most reliable and efficient regeneration systems in barley (Kuhlmann and Foroughi-Wehr, 1989). Transformation of this type of haploid and unicellular male gametophyte has the potential to generate homozygous T_0 transgenic plants when the transgenic di-haploid can be either simultaneous or chemically induced (Kuhlmann and Foroughi-Wehr, 1989). Bombardment of winter barley cv. Igri microspores with Basta® as selective agent yielded fertile and homozygous transgenic lines, but with extremely low TF (one transgenic plant in 10^7 bombarded microspores) (Jähne *et al.,* 1994); similarly, among six generated transgenic barley plants, only two were reported diploid and fertile (Yao *et al.,* 1997).

Pollen transformation by means of the *Agrobacterium* superbinary vector system and 50 μM hygromycin as selection could be achieved with an average of 2.2 fertile transgenic plants per spike (15 caryopes per spike) (Kumlehn *et al*., 2006). Approximately 60% of the T_0 transgenic plants set seeds, suggesting that spontaneous doubling happened with high rate and homozygous transgenes were detected also among the generated transgenic plants (Kumlehn *et al.,* 2006). Acetosyringone (0.5 mM) could limit the *Agrobacterium* overgrowth in the culture, but increased its transformation activity (as indicated by the *gus* expression level 1 week after pollen infection). Hygromycin was more effective than bialaphos as selection marker, suggesting that the TF could be influenced also by other factors, such as *Agrobacterium* strain/vector, medium pH, and $CaCl_2$ and glutamine concentrations (Kumlehn *et al.,* 2006).

The use of young ovules isolated shortly after pollination has been investigated as well in cultivar Golden Promise (Holme *et al.,* 2006). A TF of 3.1% (calculated as the percentage of generated transgenic plants among the total infected ovules) was obtained by hygromycin selection (50 mg/L) and the *Agrobacterium* standard binary vector system. Compared to the IE-based transformation, this system is faster, requiring only 2-3 weeks of culture before regeneration instead of 6-8 weeks for IEs. Because of the reduced *in vitro* culture time, transgenic plants produced from this system were of high quality (Holme *et al.,* 2006). This method was later extended to four other cultivars that gave a tissue culture response similar to that of Golden Promise (Holme *et al.,* 2008), implying that *Agrobacterium*-mediated, genotype independent transformation may be achieved in barley by *in vitro* cultured ovules.

Sorghum (*Sorghum* sp.)

Sorghum ranks as the fifth major cereal in this world and plays a particular role in renewable energy (Belton and Taylor, 2004). Sorghum is an important food grain in Asia and Africa because it is a drought-tolerant crop, whereas it is also an important biofuel source in America and Australia (Liu and Godwin, 2012). The worldwide sorghum yield has dramatically increased from 8896 Hg/Ha (in 1961) to 15274 Hg/Ha (in 2011) (FAO, 2013).

Sorghum is one of most recalcitrant cereal crops for *in vitro* manipulation and genetic transformation (Shrawat and Lörz, 2006). The first successful sorghum transformation was published in 1993 for a public cultivar (P898012) (Casas *et al.,* 1993). The *gus* and

bar genes were bombarded into IEs (12-15 days after pollination) with the biolistic gun PDS1000/He system. Bombarded tissues were selected on media containing 1-3 mg/L bialaphos depending on their growth stages. Of the eight genotypes tested, only three produced embryogenic calli and one produced regenerable and fertile transgenic plants. Two independent events were identified and confirmed by Southern analysis from a total of 600 bombarded embryos (Casas *et al.,* 1993).

Agrobacterium-mediated sorghum transformation was not reported until 2000, when transgenic sorghum plants were generated successfully with the public inbred P898012 and a commercial inbred (PHI391) (Zhao *et al*., 2000). IEs (9–12 days after pollination) were infected with the *Agrobacterium* strain LBA4404 carrying the superbinary vector system containing the maize ubiquitin promoter driving the *bar* or *gus* gene (Zhao *et al.,* 2000). The transformants were confirmed by Southern blot analysis in T_0 plants and the TF was 2.12% (Zhao *et al.,* 2000). The TF from the field-grown embryos was higher than that from the greenhouse-grown ones, ranging from 7.4-10.1% versus 0.95-2.17%, respectively (Zhao *et al.,* 2000).

Later, two inbred (Tx430 and C401) and one hybrid (Pioneer 8505) genotypes could be transformed by means of *Agrobacterium* standard binary vector and achieved an average TF of 2.5% (Gao *et al*., 2005a). IEs were harvested and isolated 10-14 days post anthesis and only the visual marker gene *gfp* was used to identify stably transformed tissues. By using the *pmi* gene as the selectable marker and mannose as selective agent, a TF of 3.3% and 2.88% was obtained for inbred C401 and hybrid Pioneer 8505, respectively (Gao *et al.,* 2005b).

Callus formation from IEs of the *Sorghum bicolor* cultivar Sensako 85/1191 could be improved when the embryos (1.0-1.2 mm) were dissected from immature seeds pretreated at 4°C for one day (Nguyen *et al*., 2007). A total of 15 transgenic plants were produced from 300 initial IEs (5% frequency) and confirmed by Southern blot (Nguyen *et al.,* 2007). The TF could be enhanced from 2.6% to 7.6% by heating the IEs of cultivar P898012 at 43°C for 3 min with cooling at 25°C prior to the *Agrobacterium* infection (Gurel *et al.,* 2009). In this optimized protocol with the *pmi*/mannose selection system, a TF of 7% (numbers of independent transgenic events confirmed by molecular analysis per 100 IEs infected) could be routinely achieved.

As for the other cereals, genotypes and tissue culture responses are critical factors for the success of the sorghum transformation. Sorghum genotypes, such as 296B, C401, SA281, P898012, Pioneer 8505, and Tx430, have been studied and used for generating transgenic sorghum (Sato *et al.,* 2004; Gao *et al.,* 2005b; Liu and Godwin, 2012). The most efficient explants for sorghum tissue transformation were IEs (Elkonin and Pakhomova, 2000; Gurel *et al.,* 2009; Grootboom *et al.,* 2010). A modified Murashige and Skoog (MS) medium (containing 16% more total nitrogen and 7-fold more potassium phosphate than the original formulation) was more effective for embryogenesis induction (Elkonin and Pakhomova, 2000; Sato *et al.,* 2004). Furthermore, a high level of copper (2 mM) in the callus induction and regeneration media could improve the callus induction and regeneration ratios (Nirwan and Kothari, 2003).

Recently, a highly efficient biolistic method has been reported with the particle inflow gun and with *npt*II/G418 (30 mg/L) as selection system for cultivar Tx430 (Liu and Godwin, 2012). Thanks to the systematic optimization of callus induction, regeneration, and rooting media by including and adjusting components, such as $CuSO_4$, KH_2PO_4, L-proline, L-asparagine, 2,4-dichlorophenoxyacetic acid, indole-3-acetic acid, indole-3-butyric acid, naphthalene acetic acid, and BAP, an average TF of 20.7% could be achieved from three separate experiments (Liu and Godwin, 2012), a value that may be the highest for sorghum reported from a public institution until now.

Oat (*Avena sativa*)

Oat is one of the oldest crops cultivated by humans (Lásztity, 1998) and is also the only cereal containing globulin or legume-like proteins. Its genetic diversity is high, varying from hexaploid, tetraploid to diploid, among which the hexaploid oat, *Avena sativa*, is the most cultivated and mainly adapted to temperate climates, as in Europe. Oats have been mainly used for animal feed and are now considered as health food for their cholesterol-decreasing quality (Othman *et al.,* 2011). Although due to a dramatic reduction in planted areas, the acreage decreased to only half that of 50 years ago, the yield has doubled from 12961 Hg/Ha in 1961 to 23000 Hg/Ha in 2011 (FAO, 2013). In 2011, the world's largest oat producer was Russia, followed by Canada and Australia (FAO, 2013).

Genetic transformation of oat has been accomplished mainly through bombardment. The first transgenic oat plants were generated by bombardment of IE-derived callus cultures of the oat genotype GAF-30/Park with a plasmid carrying both the *bar* and *gus* genes (Somers *et al*., 1992). In total, 111 transformants were obtained from 58 bombardments (1.9 transformant per bombardment) and 34% of the transformants regenerated into plants. However, one concern was that herbicide-resistant oat could lead to herbicide-resistant weeds when the transgenic oat was grown in the open field because oat can cross-pollinate with a wide range of relatives (Somers *et al.,* 1992). Therefore, antibiotics selective markers were tested. For selection with kanamycin, neither the *nptII*/kanamycin nor *npt*II/G418 combination was successful (Somers *et al.,* 1992; Torbert *et al.,* 1995), but the *nptII*/paromomycin combination was effective (Torbert *et al*., 1995). The IE-derived callus cultures used for bombardment provided an average of 3.1 resistant callus lines per bombardment. Almost all the paromomycin-resistant lines produced the NPTII protein as detected by enzyme-linked immunosorbent assay (ELISA) and contained the transgenes as indicated by Southern hybridization. Approximately 36% of the transgenic lines were regenerable (Torbert *et al.,* 1995), a percentage comparable with that obtained with PPT as selective agent (Somers *et al*., 1992).

Investigation of hygromycin as a selective agent revealed that IE-derived callus from oat variety Melys was less sensitive to hygromycin than to PPT (Kuai *et al.,* 2001). The use of visual markers only, such as the *gfp* gene, was tested as well (Kaeppler *et al*., 2000). Embryogenic callus of the oat genotype GP-1 was bombarded with a plasmid containing only the *gfp* gene and selected visually only for *gfp* expression. Out of 28 bombarded plates from four separate experiments, a total of 115 fertile transgenic oat plants were obtained, representing 11 independent stable *gfp*-expressing lines (Kaeppler *et al.,* 2000).

In addition to IE-derived callus tissues, explants, including mature embryos (Torbert *et al.,* 1998; Cho *et al.,* 1999; Kaeppler *et al.,* 2000), leaf base segments (Gless *et al.,* 1998), and shoot meristematic cultures (SMCs) (Zhang *et al.,* 1999), have been reported for biolistic transformation. Plasmid DNA carrying the

*npt*II and *gus* genes was bombarded into 8- to 9-week-old mature embryo-derived callus cultures of the oat genotype GAF/Park-1 (Torbert *et al.*, 1998). The bombarded callus cultures were selected on media containing 50 mg/L paromomycin and the resistant clones were regenerated after 8-10 weeks. On average, 3.2 resistant lines per bombardment were obtained, a result comparable to that from IEs as explants. A similar TF has been reported for mature embryos as explants (Kaeppler *et al.*, 2000).

Oat is the first cereal crop that could be transformed by using leaf base segments as explants (Gless *et al.,* 1998). The freshly isolated leaf base segments from two oat cultivars (Jumbo and Fuchs) and bombarded with a plasmid carrying both the *uidA* (*gus* gene from *Escherichia coli*) and *pat* genes. The bombarded tissues were cultured for 2 weeks on somatic embryogenesis-inducing medium; the embryogenic calli were selected on shoot-inducing medium containing 2 mg/L PPT. One advantage of this system is its short duration: only 10-12 weeks are required from leaf base isolation to transfer of transgenic plants to soil compared to over 12 months and 6 months needed for immature and mature embryo-derived callus as explants, respectively. Furthermore, the TF was as high as 5% (Gless *et al.,* 1998).

An efficient regeneration system was established for oat SMCs (Zhang *et al.,* 1996) with the successful generation of transgenic oat plants (Zhang *et al.,* 1999). Six-month-old SMCs from the vegetative shoots of germinating seedlings of oat cultivar Garry (Zhang *et al.,* 1998) were bombarded and selected with 2 mg/L bialaphos. Of a total of 61 transgenic plants, seven lines were resistant against bialaphos, of which 71% were fertile, a TF higher than that obtained from embryogenic callus and leaf base segments. The high regenerability and ratio of fertile plants may be due to the fact that the SMCs did not go through callus or dedifferentiation stages that usually decrease the regenerability and increase the somatic variations (Zhang *et al.,* 1999). Oat SMCs could be maintained in culture for up to 15 months without morphological changes and loss of proliferation or regeneration abilities (Zhang *et al.,* 1996, 1999). Moreover, an osmotic stress-resistant gene (the late embryogenesis abundant *hva1* gene) introduced biolistically into the SMCs of three oat cultivars (Ogle, Pacer, and Prairie) used as explants and bioalaphos as selective agent, resulted in transgenic oat plants, of which the progenies stably expressed the transgenes and their osmotic stress tolerance had significantly increased (Maqbool *et al.,* 2009). Among the above-mentioned explants, SMCs are considered superior because of their low genotype dependence in tissue culture responses, enhanced regenerability, high fertility, and small somatic variations in the produced transgenic lines (Zhang *et al.,* 1999; Maqbool *et al.,* 2002); therefore, SMCs have the potential to replace other explants for biolistic gene delivery in oat.

Only a few studies for *Agrobacterium*-mediated transformation in oat have been reported so far. Two types of explants (IEs and leaf base segments) from three oat cultivars (Bajka, Slawko, and Akt) were evaluated with the superbinary system LBA4404/pTOK233 and 50 mg/L kanamycin as selective agent and the standard binary system AGL1/pGreen and 2 mg/L PPT (Gasparis *et al.*, 2008). The TF was estimated as the percentage of selected, independent callus lines (each from one explant) that gave rise to at least one fertile transgenic plant. The IEs seemed to be more amenable to *Agrobacterium* infection and regeneration than the differentiated leaf base segments. A TF as high as 12.3% was obtained for cultivar Bajka with the LBA4404/pTOK233 system and kanamycin selection. Southern analysis on selected events revealed that 1-3 transgene copies occurred in the IE-derived transgenic plants (Gasparis *et al.,* 2008).

Millets

Millet crops are a group of small-seeded grasses, including pearl millet (*Pennisetum glaucum*), finger millet (*Eleusine coracana*), kodo millet (*Paspalum scrobiculatum*), foxtail millet (*Setaria italica*), little millet (*Panicum sumatrense*), elephant grass (*Pennisetum purpureum*), guinea grass (*Panicum maximum*), and others. Millets are widely planted in the world as staple human food, especially in the semi-arid tropical area of Africa and Asia (O'Kennedy *et al.*, 2006). The worldwide millet yield has increased from 5925 Hg/Ha (in 1961) to 8677 Hg/Ha (in 2011) (FAO, 2013). Millets, such as pearl millet, are usually tolerant to drought, because of their rapid growth, short life circle, high temperature tolerance, and deep root system (O'Kennedy *et al.,* 2009). Thus, millets are important crops in developing countries, especially with dry and high temperature climates.

The first attempt to transform millets was as early as 1987 (Hauptmann *et al.,* 1987). Protoplasts of *Pennisetum purpureum* and *Panicum maximum* were electroporated with plasmids carrying a chloramphenicol acetyltransferase gene, of which the transient expression could be detected (Hauptmann *et al.,* 1987). Because pearl millet is one of the most widely grown millets, a number of key publications were reported on this species. In 1991, by means of the biolistic method (Taylor and Vasil, 1991), IEs of pearl millet were bombarded with a plasmid carrying the *gus* gene driven by the promoter of the maize alcohol dehydrogenase gene. Transient GUS assays confirmed the DNA delivery.

Pearl millet was stably transformed with a laboratory-made particle accelerator device and tungsten particles (Lambé *et al.,* 1995). Shoot apex-derived embryogenic callus cultures or embryonic cell suspension cultures were cobombarded with two plasmids (one carrying the *gus* gene and the other the *hpt* gene) (Lambé *et al.,* 1995). The calli that survived the hygromycin (50 mg/L) selection were analyzed by Southern hybridization, GUS histochemical assays, and hygromycin phosphotransferase enzyme assays, but no plants were regenerated (Lambé *et al.,* 1995).

In 2002, transgenic pearl millet plants were generated by means of the biolistic method and IEs (Girgi *et al.,* 2002). Two biolistic delivery systems (BioRad PDS1000/He and particle inflow gun) were compared on IEs from four pearl millet genotypes that were harvested 10-14 days after pollination, dissected, precultured in callus-inducing medium for 5 days, and bombarded with plasmids carrying the *bar* and *gus* genes. The bombarded IEs were first selected on media with 1-2 mg/L bialaphos and then cultured on regeneration medium with a reduced bialaphos concentration (0.1 mg/L). The herbicide-resistant plants were confirmed by expression of the *gus* reporter genes and by Southern blot analysis in the T_0 and T_1 generations. The TFs of the particle inflow gun and BioRad particle delivery system were 0.02% (one transgenic line out of 5220 bombarded IEs of three genotypes) and 0.18% (four transgenic lines out of 2165 bombarded IEs of three genotypes), respectively (Girgi *et al.,* 2002).

Fertile transgenic pearl millet plants were obtained from a diploid hybrid HGM100 and a partial inbred tetraploid IA4X with the biolistic system and *bar*/PPT selection. Tissue culture responses and TFs

were compared from three different explant sources: IEs, inflorescences, and apical meristems. Although herbicide-resistant plants were recovered from all three types of materials, inflorescences of the tetraploid genotype seemed to have an additional advantage because of its ability to generate large quantities of targeting tissues. From 52 bombarded inflorescence-derived callus plates, 55% produced transgenic plants (155) with an average of 5.5 plants per plate. The transformation was confirmed by the reporter gene expression and Southern blot analysis (Goldman *et al.,* 2003).

The first *Agrobacterium*-mediated pearl millet transformation was not published before 2011 (Jha *et al.,* 2011). The *Agrobacterium* strain EHA105 that harbored the pCAMBIA1301 vector carrying the *hpt* and *gus* genes was used to transform shoot apices of the pearl millet inbred 843B. Shoot apices consisting of the shoot apical meristem isolated from 3- to 4-old-seedlings were incubated and vacuum treated with the *Agrobacterium* culture, cocultivated, and grown in a recovery medium (without selection) for 7 days. The infected explants were cultured in selective and shoot-inducing media containing 30 mg/L hygromycin and the surviving shoots were regenerated. Molecular analysis, such as Southern hybridization and polymerase chain reaction, confirmed that transgenic pearl millet plants could be obtained routinely with this procedure. The best TF reported was 5.79% (Jha *et al.,* 2011).

By means of a similar approach using the *Agrobacterium* strain LBA4404 harboring the pCAMBIA1301 vector and hygromycin as selection marker, two genotypes of finger millet could be transformed (Ceasar and Ignacimuthu, 2011). However, embryogenic callus derived from shoot apices were infected instead of freshly isolated shoot apices without vacuum treatment at the infection step. This optimized procedure produced stable transformation between 1-4% (Ceasar and Ignacimuthu, 2011).

Recently, foxtail millet has received some attention because it is closely related to the bioenergy grass switchgrass (Lata *et al.,* 2012). In addition, because it has a small diploid genome and an inbreeding nature, it is considered a useful experimental crop and its genome has been sequenced (Zhang *et al.,* 2012). Three types of explant (IEs, shoot apices from germinating seedlings, and inflorescences) from six local foxtail millet cultivars were tested for induction and establishment of embryogenic callus cultures (Liu *et al*., 2005). For the *Agrobacterium*-mediated transformation the LBA4404 strain, harboring a binary pBI121 vector carrying the *nptII* and *gus* genes, was used to infect inflorescence-derived calli. Plants resistant to kanamycin (50 mg/L) were confirmed by Southern hybridization done on three plants. A 5-9% TF was reported with this protocol (Liu *et al.,* 2007).

Compared to major cereal crops, such as maize, rice, and wheat, limited information on genetic transformation of millets could be found. Whereas both *Agrobacterium* and bombardment have been applied successfully in millet, the biolistic gun is still the dominant method. For a more comprehensive and detailed review on millet tissue culture and transformation, we refer the reader to Ceasar and Ignacimuthu (2009) and Plaza-Wüthrich and Tadele (2012).

Conclusion

The two most efficient and widely used methods for genetic transformation are the delivery systems mediated by *Agrobacterium* and particle bombardment. *Agrobacterium tumefaciens* is a soil bacterium known to cause crown gall disease in plants. The unique feature of this pathogen is its ability to deliver and integrate part of its own DNA (T-DNA) into the genome of the infected cells. However, the limitation of this system lies in the susceptibility of the host plant to *Agrobacterium* and is, hence, highly dependent on plant and genotype. In contrast, transformation via bombardment is a physical delivery method based on the rapid acceleration of DNA-coated metal microprojectiles into target cells. Therefore, it can transform a broader spectrum of plant species and genotypes as long as the target tissue is regenerable. Since its development in mid-1980s (Klein *et al.,* 1987), biolistic bombardment has been used to transform almost all major cereal crops, including maize, rice, wheat, barley, and sorghum. The disadvantages of the biolistic delivery are its complex integration patterns and high-copy numbers of transgenes in the transgenic plants, often leading to gene silencing and unstable inheritance (Dai *et al.,* 2001; Hu *et al.,* 2003; Shou *et al.,* 2004; Travella *et al.,* 2005). Currently, if a plant could be transformed by both methods, the *Agrobacterium* method would be chosen to obtain high numbers of single or low-copy transgene insertion. Nevertheless, both methods deliver DNA into the genome at random positions. The *Agrobacterium*-mediated method also generates transgenic events that carry DNA fragments outside the T-DNA region (vector backbones) (Shou *et al.,* 2004), which is an undesired effect. To prevent extra DNA segments from being inserted into the genome, some researchers used purified DNA fragments that carry only the cassette with the gene of interest for bombardment (Breitler *et al.,* 2002; Loc *et al.,* 2002), whereas others devised multiple T-DNA borders (Kuraya *et al.,* 2004) or launched T-DNA from the *Agrobacterium* chromosome (Oltmanns *et al.,* 2010) to reduce the backbone insertion

TABLE 1

SUMMARY OF TRANSFORMATION PROTOCOLS WITH HIGHEST FREQUENCIES

Crops	Explants	Selection system Gene/agent* (mg/L)	Delivery method	Transformation frequency (%)	References
Maize	IE	*bar*/PPT (10)	*Agrobacterium*	50; T_0 PPT-resistant and GUS-positive transgenic plants	Ishida *et al.* (2007)
Rice	Mature embryo-derived callus	*htp*/Hm (50)	*Agrobacterium*	92; Hm-resistant, GUS-positive transgenic plants	Ozawa (2009)
	Mature embryo-derived callus; IE	*htp*/Hm (50)	*Agrobacterium*	50-90; Independent T_0 events	Hiei and Komari (2008)
Wheat	Precultured IE	*pmi*/mannose (5)	Biolistic	20; Independent T_0 events	Wright *et al.* (2001)
	IE-derived callus	*nptII*/G418 (25)	*Agrobacterium*	10.5; Independent T_0 events, validated by Southern blot	Cheng *et al.* (2003)
Barley	IE	*htp*/Hm (50)	*Agrobacterium*	25; Independent Hm-resistantT_0 events	Bartlett *et al.* (2008)
Sorghum	IE	*nptII*/G418 (30)	Biolistic	20.7; Independent T_0 events	Liu and Goodwin (2012)
Oat	IE	*nptII*/Km (50)	*Agrobacterium*	12.3; Independent T0 events	Gasparis *et al.* (2008)
Millet	Inflorescence-derived callus	*nptII*/Km (50)	*Agrobacterium*	5-9; T_0 transgenic plants, GUS-positive and Southern blot	Liu *et al.* (2007)

*Hm, hygromycin; Km, kanamycin.

frequency. In the future, both methods will probably be needed to maximize the genetic transformation of plants. Table 1 summarizes a few protocols that gave the highest transformation frequencies for seven cereal crops.

Thirty years ago, the world witnessed the first transgenic plant produced by the *Agrobacterium tumefaciens*-mediated method (Herrera-Estrella *et al*., 1983). Via various delivery systems, especially the *Agrobacterium*-mediated and biolistic gun methods, most plants, including all major cereal crops, can be genetically engineered. The technology becomes more efficient and applicable to an ever increasing number of plant species and cultivars. Plant genetic transformation technology has changed the way to study plants and grow crops. We believe that its continuous improvement will have a very positive impact on our society and environment.

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